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# Studies on Anti-tuberculous Low Molecular Factors in Various Organs of Rabbits

## Part I. Studies on crude materials

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### INTRODUCTION

The problems of resistance of various animals to tuberculous infection have been investigated from the viewpoint of the function of cells[1)2)3)] or of body fluid[4)5)]. In general, although the function of cells has been thought to be more significant in the defense mechanism of animals against tuberculosis than that of fluids, it has been recently demonstrated by our associates that body fluids.... human urine and serum of various animal species.....contains powerful tuberculostatic agents and that these agents may play a very important role in the mechanism of noticeable resistance of animals[6)].

In our laboratory, using several new experimental methods, (the slide culture method[7)], the chamber method[8)], and the ring method[9)],) the resistant factors of animal body fluids to tuberculous infection have been investigated and analysed. It has been demonstrated that the low molecular substances (able to pass through a cellophane membrane) in animal body fluid can act to inhibit and the high molecular substances to promote the growth of virulent tubercle bacilli. It has been proved that there are some substances in human urine or in the serum of various animal species which can inhibit the growth of tubercle bacilli *in vitro*.

And it has also been demonstrated that active substances may be some sorts of amino acids, peptides or organic acids[10)].

Subsequent investigations dealing with the tuberculostatic factors of various organ extracts of normal or immune rabbits, will be reported in this paper. In a series of investigations, this first part is concerned with crude materials of various organ extracts of rabbits.

### MATERIALS AND METHODS

#### Preparation of materials

*Preparing of rabbit's organ extracts* : A rabbit weighing 3 kg was bled to

death by cutting the neck artery. To promote blood flow, 1 ml of heparin solution (1,000 units) had been previously injected into an auricular vein.

Each organ (muscle, liver, kidney, brain and lung) was washed with physiologic saline solution through its artery and vein by pumping to remove as much blood as possible until the washing water became perfectly blood free. The spleen, however, could not be washed thus, because vessels were too thin to insert an injection needle. The muscle was collected from the femoral muscles. All washed organs were weighed and recorded. After being cut grossly with scissors, the organ tissue was homogenized for one or two minutes by adding an adequate amount of physiological saline solution (the amount is different for each organ : the liver, kidney and brain could be homogenized without the solution, but the muscle and lung needed the solution, as much as half the volume of the original material.) The homogenized organ was enclosed in a cellophane membrane (for dialysis No. 300) and dialysed in fifteen- or twenty-fold volumes of sterile distilled water at 4°C for 72 hours.

The outer fluid produced by dialyzing was concentrated under reduced pressure (less than 10 mg Hg of pressure) and at 50°C in temperature to dryness. The dried materials looked like yellowish-brown caramel containing some crystals, and were deliquescent. The color of the dried materials changed easily from yellowish-brown to dark-brown at about 60°C in temperature. These dried materials are designated as "the crude materials" of organ extracts. The yield of the crude material was not uniform according to the organ or the animal used.

*Immunization* : Using the same procedure, we also prepared crude materials from immunized rabbits. Normal rabbits weighing 3 kg were injected with 100 mg heat-killed H37Rv strain or 50 mg living BCG suspended in paraffin oil into the gluteal muscle twice with an interval of two weeks. Two weeks after the last injection, the rabbit showed a positive skin reaction to the ten-fold diluted old tuberculin.

**Culture method**

*Preparation of medium* : The dried "crude materials" of organ extracts of rabbits were dissolved in 1/8 the volume of the starting organ materials with distilled water.

This solution was called "eight-fold concentrated solution of materials".

*Test for tuberculostatic activity* : The slide culture method was used. Two milliliters of Kirchner's liquid, containing the serially diluted "crude materials", was prepared. The composition of one of the serial media is shown in Table 1. Slides which were smeared with the test bacteria (H37Rv strain, unless otherwise noted) and treated by the benzine method were immersed in the media. Cultures were continued for ten days at 37°C.

Table 1. The Series of Cultur Medium.

| Component | Concentration | | | | | | |
|---|---|---|---|---|---|---|---|
| | 8× | 4× | 2× | 1× | 1/2× | 1/4× | C |
| Crude material (8×) | 1.6 | 0.8 | 0.4 | 0.2 | 0.1 | 0.05 | 0 |
| Distilled water | 0 | 0.8 | 1.2 | 1.4 | 1.5 | 1.55 | 1.6 |
| Kirchner's Liquid (10×) | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Bovine Albumin | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

"1×" means the same concentration as the orginal organ, and "2×" means the two-fold concentration.

The degree of growth of bacilli is designated as follows :

+++ Colonies abhere to each other, and there are no distinct colonies.

++ Very good proliferation, but there are distinct colonies

\+ Good proliferation (by oil immersion)

− No bacillary growth.

Using the method described above, several strains of mycobacteria were cultured and the growth-inhibiting action of the added "crude materials" from various organs of normal or immunized rabbits was examined. Furthermore, stability against heat or hydrolysis, and solubility in several organic solvents of the effective factors in the crude materials were also examined.

## RESULTS

### 1. The inhibitory effect of the crude materials on the growth of the H37Rv strain.

a) The crude materials from the normal rabbits :

The results are shown in Table 2. It can be seen that while the muscle and the liver showed an apparent inhibitory effect on the growth of the H37Rv strain at a "1×" concentration (i.e., the same concentration as in the living state), the lung and the spleen could not inhibit at a two-fold concentration ("2×") of the starting organs. The brain and the kidney sometimes could and sometimes could not inhibit at a two-fold concentration.

The relation, in one case, between the inhibitory effect and the calculated amount (mg) of the crude material in Kirchner's medium (1 ml) is shown in Table 3 and Fig. 1. The smallest amount capable of inhibiting the growth of bacilli was 16 mg in the muscle, 24 mg in the liver, 40 mg in the brain and kidney, but in the lung and the spleen, the smallest amount could not be determined in this case.

Of course, there are individual differences in the inhibiting ability of ani-

Table 2. The effect of the crude materials of several organ extract of normal rabbit on the growth of tubercle bacilli.

| Organ | Concentration | | | | | |
|---|---|---|---|---|---|---|
| | 8× | 4× | 2× | 1× | 1/2× | 1/4× |
| Muscle | −<br>− | −<br>− | −<br>− | −<br>− | −<br>+++ | ++<br>+++ |
| Liver | −<br>− | −<br>− | −<br>− | −<br>− | +<br>++ | ++<br>++ |
| Kidney | | −<br>− | −<br>− | +<br>++ | ++<br>++ | +++<br>+++ |
| Brain | | | −<br>++ | −<br>++ | +<br>+++ | +++<br>+++ |
| Lung | | | +<br>++ | ++<br>+++ | +++<br>+++ | +++<br>+++ |
| Spleen | | | +<br>+ | ++<br>++ | ++<br>+++ | +++<br>+++ |

Control +++
Slide culture using H37Rv strain in Kirchner's medium for ten days

Table 3. The relation between the effect and the amount of the crude material in Kirchner's medium on the growth of H37Rv strain.

| Organs | Dose of materials (mg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 40 | 35 | 30 | 25 | 20 | 15 | 10 | 5 | 0 |
| Muscle | − | − | − | − | − | − | | +++ | +++ |
| Liver | − | − | − | − | | | ++ | +++ | +++ |
| Kidney | − | | | | ++ | ++ | +++ | +++ | +++ |
| Brain | − | | | | ++ | ++ | +++ | +++ | +++ |
| Lung | | | | ++ | ++ | +++ | +++ | +++ | +++ |
| Spleen | | | ++ | ++ | ++ | +++ | +++ | +++ | +++ |

mals, and the absolute value of the inhibiting dose of the crude material is not uniform in all experiments, but there is always a distinct relationship between the inhibiting ability and the organs. The grade of inhibiting ability is in the following descending order.....muscle, liver, brain, kidney, lung and spleen.

b) Crude materials from immunized rabbits :

The crude materials of the various organ extracts of rabbits which were im-

munized by 100 mg heat-killed H37Rv strain or 50 mg living BCG were examined by the procedure described above. The results in one case are shown in Table 4 and no significant difference between the data obtained from the animals immunized by killed bacilli or by living bacilli was noted. Although the inhibiting ability of muscle and liver is almost the same as that obtained in normal rabbits, it can be noted that the other organs have a slightly more powerful effect on the growth of bacilli than those obtained from normal rabbits. But, as there is a problem of individual variation, comparing these three (normal and two immunized) experimental results, it is almost certain that the degree of growth-inhibiting power of various immure organs has not altered from that of normal organs.

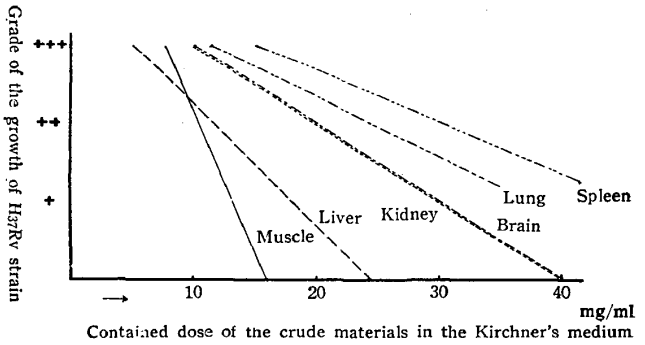


Fig. 1. The relation between the amount of the crude materials (mg) in Kirchner's memium and their effect on the growth of the H37Rv strain.

Table 4. The effect of the crude materials of several organ extract from the immunized rabbits on the growth of tubercle bacilli.

The rabbits were immunized with 100 mg heat killed H37Rv strain and 50 mg living BCG suspended in paraffin oil injected into the gluteal muscle twice with an interval of two weeks.

| Organs Immunized | | Concentration | | | | | |
|---|---|---|---|---|---|---|---|
| | | 8× | 4× | 2× | 1× | 1/2× | 1/4× |
| Muscle | H37Rv | − | − | − | − | ++ | +++ |
| | BCG | − | − | − | − | ++ | +++ |
| Liver | H37Rv | − | − | − | − | + | +++ |
| | BCG | − | − | − | − | ++ | +++ |
| Kidney | H37Rv | | − | − | + | +++ | +++ |
| | BCG | | − | − | ++ | +++ | +++ |
| Brain | H37Rv | | | − | + | +++ | +++ |
| | BCG | | | − | ++ | +++ | +++ |
| Lung | H37Rv | | | + | ++ | +++ | +++ |
| | BCG | | | + | ++ | +++ | +++ |

Control +++

Slide culture using H37Rv stain in Kinchner's medium for ten days.

## 2. Heat stability

Sterilization is indispensable in the process by which the antibacterial effect of these crude materials is examined. So it is necessary to examine how heat stable these crude materials are. For this purpose, the temperature for sterilization was changed as follows.

i. Sterization by Seitz's filter (no heating)
ii. Sterilization by heating at 100°C for 30 min.
iii. Sterilization by heating at 120°C for 30 min.

These experimental results are shown in Table 5. These crude materials are so stable to heat that we usually can use sterilization by heat at 100°C for 30 min.

Table 5. Heat stability of the crude material of muscle extract from the normal rabbit.

| Sterilization | Concentration | | | | |
|---|---|---|---|---|---|
| | 4× | 2× | 1× | 1/2× | 1/4× |
| Filtration with SEITZ's Filter | − | − | − | +++ | +++ |
| Heating 100°C for 30 min. | − | − | − | +++ | +++ |
| Heating 120°C for 30 min. | − | − | − | +++ | +++ |

Controle +++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

## 3. The solubility in several organic solvents of the crude materials of organ extracts and their tuberculostatic effects.

The solubility of the crude materials of organ extracts in several organic solvents was examined. As solvents, methanol, ethanol, ethyl ether and acetone were used.

After the crude materials had been warmed and evaporated to dryness at 50-60°C, 50-100 ml of solvent was added to the dry material.

After being stirred and shaken the soluble and insoluble portions were separated by filtration or decantation. Both portions were again warmed and evaporated to dryness in order to remove solvents and were redissolved in distilled water. After sterilization, at 100°C for 30 min., the tuberculostatic activity was tested.

In this case, we used only muscle and liver as testing materials, because the other organs were too small in amount to get a large enough volume for the examinations.

The results are shown in Table 6. It can be seen that the active portions are soluble in water and methanol, while they are only very slightly soluble or entirely insoluble in ethanol, ethyl ether and acetone.

Table 6. The solubility in organic solvents of the crude material of muscle from the normal rabbit and their effect on the growth of H37Rv strain.

| Solvents | Parts Soluble or Insoluble | Concentration | | | | |
|---|---|---|---|---|---|---|
| | | 4× | 2× | 1× | 1/2× | 1/4× |
| Water | soluble | − | − | − | ++ | +++ |
| Methanol | soluble | − | − | − | ++ | +++ |
| | insoluble | + | ++ | +++ | +++ | +++ |
| Ethanol | soluble | ++ | ++ | +++ | +++ | +++ |
| | insoluble | − | − | − | ++ | +++ |
| Ether | soluble | ++ | +++ | +++ | +++ | +++ |
| | insoluble | − | − | − | ++ | +++ |
| Acetone | soluble | ++ | ++ | +++ | +++ | +++ |
| | insoluble | − | − | − | ++ | ++ |

Control +++
Slide culture in Kirchner's medium for ten days.

**4. Chemical properties examined in these crude materials.**

The following chemical reactions were examined using these crude materials.

| Reaction | Results | Meanings |
|---|---|---|
| Sulfosalicilic acid R. | (−) | No proteins |
| Ninhydrin R. | (+) | Amino acids present |
| Millon's R. | (+) | Amino acids present |
| Jaffe's R. | (+) | Creatinine presents |
| Molisch's R. | (+) | Active reducing substances present |
| Nylander R. | (+) | Active reducing substances present |

These results may indicate that these crude materials are really low molecular and containing no protein or other high molecular substances.

## DISCUSSION

We have proved that there are substances, which can inhibit the growth of tubercle bacilli *in vitro* in various organs (muscle, liver, kidney, lung and spleen) in rabbits. These substances are low molecular substances which are able to pass through a cellophane membrane. The grade of the inhibiting activity is different according to the organ, the substances from muscle being strongest, followed by, in descending order, liver, kidney and brain, lung and spleen.

These substances are heat stable and their activity is not decreased even by heating at 120°C for 30 min. or by hydrolysis with heating with 6N HCl in

boiling water for 15 hours[33]. They are easily soluble in water and methanol but almost insoluble in ethanol, ethyl ether or acetone. It may be worthy of note that the activities of these substances did not increase after immunization, that is, that tuberculous immunity is not conferred by these low molecular antituberculous substances in organs. These characteristics of the substances in various organ extracts of rabbits which can inhibit the growth of tubercle bacilli *in vitro* are quite similar to the tuberculostatic substances which were previously found by Fujita[10][11] in the low molecular substances of rabbit's serum. Oshima[10][12] also proved the presence of tuberculostatic substances in human urine. But the activity in urine is not so stable to heat or hydrohysis as that in organ extract or serum, so it seems to us that there are some differences between the effective substances of organ extracts or serum and those in human urine.

Previously, Björnesjö[13]-[20] reported the presence of tuberculostatic substances in human urine, and also the presence of similar substances in the bovine spleen, kidney, lung, liver and muscle. He stated, however, that the muscle substance was the weakest in activity as compared with that of other organs. That fact is opposite to our finding that muscle is the most active.

Diehl[21] showed that tubercle bacilli which were incubated with the organ emulsions were inhibited either in their power to infect guinea pigs or in their growth on Hohn's medium. He implied that this fact demonstrated the presence of some tuberculocidal substances.

Ozasa[22] examined the tuberculostatic effect of alcoholic extracts of lung, liver, spleen and kidney of guinea pigs and found they contained substances which inhibited the growth of tubercle bacilli, while the other organs had growth promoting substances.

The contradictory results reported by the last-mentioned two authors, may be resolved if we consider that the substances should be divided into two categories, low and high molecular substances. As a matter of fact, it has been demonstrated by our recent investigations that the low molecular substances in the serum of various animals act to inhibit and the high molecular substances to promote the growth of tubercle bacilli. Therefore, it may be inadequate to deal with both high and low molecular substances together in examining the growth inhibiting activity.

Dubos et al.[23][24] demonstrated fairly pure substances in several organs, that is spermin in the kidney and thymus-peptide in the calf thymus as antituberculous factors in the organs.

There are some other investigations dealing with antituberculous substances in various chemical factors in animal organs, in serum, in urine, and even in milk[25]-[32].

In short, although it may be natural that there are many substances which

are capable of acting on the growth of tubercle bacilli in so complicated a substance as one taken from an organ extract, it may still be noteworthy that no distinct relationship exists between these substances and the organ disposition to tuberculosis. On the other hand, in this investigation we can demonstrate a somewhat reasonable parallelism between the antituberculous activity of an organ substance and the resistance of these organs to tuberculosis.

## SUMMARY

1. The tuberculostatic activity of low molecular substances (able to pass through a cellophane membrane) in various organ extracts, (muscle, liver, kidney, brain, lung and spleen) of rabbits was investigated. And it was confirmed that substances which can inhibit *in vitro* growth of the H37Rv strain of tubercle bacilli were actually present. The activity of these substances is, in descending order, muscle (strongest), liver, kidney and brain, lung and spleen.

2. These substances are soluble in water and methanol but insoluble in ethanol, ethyl ether and acetone.

3. These substances are stable to heating at 120°C for 30 min.

4. The activity of these substances is not increased by immunization of the rabbits.

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